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<p>(54) Title: HUMAN SPASMOLYTIC POLYPEPTIDE IN GLYCOSYLATED FORM</p> <p>Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu Ser Pro His Asn Arg Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val Pro Trp Cys Phe His Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val Met Glu Val Ser Asp Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu Val Pro Trp Cys Phe Phe Pro Asn Ser Val Glu Asp Cys His Tyr (I)</p> <p>(57) Abstract</p> <p>Human spasmolytic polypeptide (HSP) which has the amino acid sequence (I) or a functionally equivalent homologue thereof, characterized by being in glycosylated form.</p>		

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HUMAN SPASMOLYTIC POLYPEPTIDE IN GLYCOSYLATED FORM

FIELD OF INVENTION

The present invention relates to human spasmolytic polypeptide in glycosylated form, variants of human and porcine spasmolytic polypeptides and a method of producing spasmolytic polypeptides in glycosylated form.

BACKGROUND OF THE INVENTION

Human spasmolytic polypeptide (HSP) belongs to a family of peptides containing one or more characteristic trefoil domains [1]. The trefoil domain is made up of a sequence of 38 or 39 amino acid residues in which 6 cystein residues are linked in the configuration 1-5, 2-4 and 3-6 thus forming a characteristic trefoil structure [1]. The trefoil family of peptides consists of rat intestinal trefoil factor, ITF [2], human breast cancer associated peptide, pS2 [3,4,5], porcine, human and murine spasmolytic polypeptide (PSP, HSP, MSP) [6,7,8] and frog spasmolysins (xP1, xP2 and xP4) [8,10,11] all containing 1, 2 or 4 trefoil domains (Fig. 1).

The physiological function of the trefoil peptides is poorly understood, and so far only PSP has been studied in any detail. In the porcine pancreas, PSP is found in the acinar cells and to be secreted in large amounts (50-100 mg/ml) into the pancreatic juice upon stimulation with pancreozymin or secretin [12,13,14]. PSP is resistant to digestion by intestinal proteases in the gastrointestinal tract [12], and specific binding of PSP to rat intestinal mucosa cells and membrane preparations from these cells has been demonstrated [15,16]. In the porcine gastrointestinal tract, specific receptor-like binding to Paneth cells in the duodenum has been found [17]. These results suggest a unique intraluminal function of the peptide. A pharmacological screening has indicated that PSP has spasmolytic and gastric acid secretion inhibitory effects [18],

and studies on mammalian cells have indicated a growth factor-like activity of PSP [19].

The DNA sequence and derived amino acid sequence of the human counterpart of porcine SP is shown in [8]. Unlike PSP, human SP 5 (Fig. 2), has been found to be expressed in the stomach, but not in the pancreas to any greater extent [8]. An increased expression of HSP and pS2 has been reported to be associated with peptic ulcers and mucosal injury in inflammatory bowel disease [20,21] indicating a possible healing function of these 10 peptides.

Only very limited amounts of HSP can be prepared by extraction of human tissue. An object of study resulting in the present invention was therefore to prepare recombinant HSP in sufficient amounts for physiological and biochemical studies of 15 the peptide.

SUMMARY OF THE INVENTION

It has surprisingly been found that when recombinant HSP is produced in certain host organisms, a proportion of it is produced in glycosylated form by posttranslational 20 modifications. The glycosylated form of HSP has not, to applicant's best knowledge, been described previously.

Accordingly, the present invention relates to human spasmolytic polypeptide (HSP) which has the amino acid sequence

Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu Ser Pro His Asn Arg
25 Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn
Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val Pro Trp Cys Phe His
Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val Met Glu Val Ser Asp
Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser
Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu Val Pro Trp Cys Phe
30 Phe Pro Asn Ser Val Glu Asp Cys His Tyr (SEQ ID NO:1)

or a functionally equivalent homologue thereof, characterized by being in glycosylated form.

In the present context, the term "functionally equivalent" is intended to indicate that the homologous polypeptide has a biological activity (e.g. spasmolytic effect) corresponding to that of native HSP. The term "homologue" is intended to indicate a polypeptide encoded by DNA which hybridizes to the same probe as the DNA coding for HSP under conditions of high or low stringency (e.g. as described in Sambrook et. al., 10 Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). More specifically, the term is intended to refer to a DNA sequence which is at least 60% homologous to the sequence encoding HSP with the amino acid sequence shown above. The term is intended 15 to include modifications of the DNA sequence such as nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide, but which correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a 20 different amino acid sequence and therefore, possibly, a different protein structure which might give rise to a mutant polypeptide with different properties than the native enzyme. Other examples of possible modifications are insertion of one or more codons into the sequence, addition of one or more 25 codons at either end of the sequence, or deletion of one or more codons at either end or within the sequence. The term "glycosylated" is intended to indicate that a carbohydrate moiety is present at one or more sites of the protein molecule.

It is at present contemplated that glycosylation of HSP may 30 give rise to differences in the biological activity of the protein, for instance with respect to stability towards proteolytic enzymes in the gastrointestinal tract, solubility at gastric and/or intestinal pH compared to non-glycosylated HSP, antigenicity, half-life, tertiary structure, and targeting 35 to receptors on appropriate cells.

In another aspect, the present invention relates to a variant of a spasmolytic polypeptide (SP) which is a fragment of human spasmolytic polypeptide (HSP) or porcine spasmolytic polypeptide (PSP) comprising at least one trefoil domain.

5 The variant SP may be provided in both glycosylated and non-glycosylated form. It is at present contemplated that such a variant may be advantageous to use instead of full-length SP because of a higher specific biological activity, increased solubility and stability, longer half-life, easier way of
10 production, or the like.

It is assumed that other spasmolytic polypeptides than HSP will, if provided with a glycosylation site, also be expressed in predominantly glycosylated form. In a further aspect, the present invention therefore relates to a method of preparing a
15 spasmolytic polypeptide in at least 60% glycosylated form, wherein a host cell transformed with a DNA fragment encoding a spasmolytic polypeptide and capable of providing glycosylation of said spasmolytic polypeptide is cultured under conditions permitting production of said spasmolytic polypeptide and
20 recovering the resulting spasmolytic polypeptide from the culture.

DETAILED DESCRIPTION OF THE INVENTION

It has been found that, at least when recombinant HSP is produced in yeast, the proportion of it that is provided in
25 glycosylated form is in N-glycosylated form. It has further been found that glycosylation takes place at Asn15 of the sequence shown above. In preferred embodiments of glycosylated HSP, the glycosylated side chain contains at least one hexose unit. In particular, the glycosylated side chain may contain at
30 least one mannose unit, preferably at least five mannose units, most preferably at least ten mannose units. In one preferred embodiment of glycosylated HSP of the invention, the glycosylated side chain contains 13-17 mannose units. In other

preferred embodiments, the glycosylated HSP is in addition glycosylated with at least one unit of N-acetyl glucosamine (GlcNAc). In the currently preferred embodiment, the glycosylated HSP is glycosylated at Asn15 with (GlcNAc)₂(Man)₁₀.

5 15.

It is further contemplated to produce homologues of HSP which are provided with one or more additional glycosylation sites. Thus, the present invention also relates to HSP homologues, wherein Lys2 is replaced by Asn, Gln7 is replaced by Asn, Arg10
10 is replaced by Asn, Gly 20 is replaced by Thr or Ser, Gly23 is replaced by Asn, Ile 24 is replaced by Asn, Phe 36 is replaced by Asn, Asp 37 is replaced by Asn, Ser39 is replaced by Asn, Gln53 is replaced by Asn, Glu61 is replaced by Asn, Asp64 is replaced by Asn, Arg66 is replaced by Thr or Ser, Gly69 is
15 replaced by Thr or Ser, Gly72 is replaced by Asn, Ile 89 is replaced by Thr or Ser, Pro98 is replaced by Asn or Val101 is replaced by Thr or Ser, or a combination of two or more of these substitutions. In a currently preferred embodiment of such an HSP homologue, Asp64 is replaced by Asn, and Arg66 is
20 replaced by Thr or Ser.

It is of course understood that HSP homologues of the invention may be glycosylated in the same manner at one or more of these sites as described above for glycosylation at Asn15.

It is assumed that the trefoil structure common among
25 spasmolytic polypeptides is important for the function of HSP and PSP. The variant human or porcine SP comprising a fragment of the full-length polypeptide should therefore include at least three disulfide bonds to provide this structure. Consequently, the variant may comprise at least a sequence of
30 amino acids from position 8 to 46 or from position 58 to 95, each of which sequences defines a trefoil domain of HSP and PSP.

As indicated above, the SP variant of the invention may be provided in non-glycosylated form. This may, for instance, be accomplished by substituting Asn15 by another amino acid, e.g. Asp or Glu, or by substituting Thr17 by another amino acid except Ser, e.g. Ala. It is more likely, however, that one or more additional glycosylation sites will be introduced into this domain, for instance by replacing Arg10 by Asn, Gly 20 by Thr or Ser, Gly23 by Asn, Ile 24 by Asn, Phe 36 by Asn, Asp 37 by Asn, or Ser39 by Asn, or a combination of two or more of these substitutions.

On the other hand, it may be desirable to provide the trefoil domain from position 58 to 95 with a glycosylation site lacking in this domain in native HSP and PSP. Thus, Glu61 may be replaced by Asn, Asp64 by Asn, Arg66 by Thr or Ser, Gly69 by Thr or Ser, or Gly72 is replaced by Asn, or a combination of two or more of these substitutions. In a currently preferred embodiment of the variant, Asp64 is replaced by Asn, and Arg66 is replaced by Thr or Ser.

It is of course understood that variants of the invention may be glycosylated in the same manner at one or more of these sites as described above for glycosylation at Asn15 in full-length HSP.

A DNA sequence encoding HSP may suitably be isolated from a human genomic DNA library by PCR (polymerase chain reaction) cloning using primers based on the published cDNA sequence [8]. Alternatively, the DNA sequence may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors. The cDNA sequence shown in [8] may be used as the basis of oligonucleotide synthesis.

Alternatively, it is possible to use cDNA coding for HSP obtained by screening a human cDNA library with oligonucleotide probes in accordance with well-known procedures.

Furthermore, the DNA sequence may be of mixed synthetic and 5 genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of genomic, synthetic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques.

10 The SP variant of the invention may be encoded by a fragment of the full-length DNA sequence, prepared by one of the methods indicated above, or by suitably truncating the full-length sequence.

The DNA sequence encoding HSP or an SP variant of the invention 15 may then be inserted in a suitable expression vector. The recombinant expression vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an 20 autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and 25 replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding HSP or an SP variant of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which 30 shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding

the inhibitor of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter.

The DNA sequence encoding HSP or an SP variant may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication, or (when the host cell is a yeast cell) the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or

methotrexate, or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130.

The procedures used to ligate the DNA sequences coding for HSP or the SP variant, the promoter and the terminator, 5 respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

10 The host cell into which the expression vector is introduced may be any cell which is capable of producing the inhibitor of the invention and is preferably a eukaryotic cell, such as a mammalian, yeast or fungal cell.

The yeast organism used as the host cell may be any yeast 15 organism which, on cultivation, produces large quantities of the inhibitor of the invention. Examples of suitable yeast organisms are strains of the yeast species Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe or Saccharomyces uvarum. The transformation of yeast cells may 20 for instance be effected by protoplast formation followed by transformation in a manner known per se.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and 25 expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and 30 Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells may be used as host cells. Examples of suitable fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 238 023.

According to the present method, yeast cells are currently preferred for producing HSP and other SPs (such as those shown in Fig. 1), as they have surprisingly been found to produce SP 10 in a high yield and in at least 60% glycosylated form. For instance, about two thirds of the HSP produced by yeast may be recovered in glycosylated form.

The medium used to cultivate the cells may be any conventional medium suitable for growing mammalian cells or fungal 15 (including yeast) cells, depending on the choice of host cell. The spasmolytic polypeptide will be secreted by the host cells to the growth medium and may be recovered therefrom by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the 20 proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography or affinity chromatography, or the like.

The present invention also relates to a pharmaceutical 25 composition comprising HSP or a variant spasmolytic polypeptide of the invention together with a pharmaceutically acceptable carrier or excipient. In the composition of the invention, the variant may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in 30 Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for oral or rectal administration and may, as such, be formulated as tablets or suppositories.

HSP or an SP variant of the invention is contemplated to be useful for the prophylaxis or treatment of gastrointestinal disorders. More specifically, it is contemplated for the treatment of gastric or peptic ulcers, inflammatory bowel disease, Crohn's disease or injury to the intestinal tract caused by radiation therapy, bacterial or other infections, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated in the following example with reference to the appended drawings in which

Fig. 1 shows the trefoil family of peptides. Intestinal trefoil factor (ITF) contains one trefoil domain [2], as does the breast cancer associated pS2 peptide [3,4]. The spasmolytic polypeptides from man, pig and mouse contain two trefoil domains [1,8]. Spasmodysins from Xenopus laevis contain one or four trefoil domains [10]. Recently, a member of the frog trefoil family containing two domains has been described [11].

Fig. 2 shows the proposed structure of human spasmolytic polypeptide, HSP. The primary amino acid sequence is taken from Tomasetto et al. [8], and the disulphide bonds are placed in homology to PSP [1].

Fig. 3 shows the nucleotide sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO: 3) of the 563 bp EcoRI - XbaI fragment encoding the leader - HSP fusion protein. The Kex 2 processing site is indicated by a vertical arrow. The leader and the PCR cloned parts of the HSP gene are shown in capital letters, while the synthetic parts are shown in small letters. The underlined sequences correspond to the PCR primers with horizontal arrows indicating the direction. Restriction sites relevant for the construction are shown.

Fig. 4 shows the S. cerevisiae plasmid for the expression and secretion of HSP. TPI-prom. and TPI-term. are S. cerevisiae triosephosphate isomerase transcription promoter and terminator sequences, respectively. POT is a selective marker, the
5 Schizosaccharomyces pombe triosephosphate isomerase gene. Only restriction sites relevant for the construction of the plasmid have been indicated.

Fig. 5 shows reversed-phase HPLC on a Vydac 214TP54 column of yeast fermentation broth. The two peaks corresponding to r-HSP
10 and glycosylated r-HSP are indicated. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Fig. 6 shows ion exchange chromatography on a Fast Flow S column of concentrated yeast supernatant. The amount of r-HSP and glycosylated r-HSP were determined by the use of the HPLC
15 system shown in Fig. 5. The bars indicate the fractions pooled for further purification of r-HSP and glycosylated r-HSP. The dashed line shows the concentration of NaCl in the eluting solvent. For details, see Material and Methods.

Fig. 7 shows the final purification of r-HSP (A) and
20 glycosylated r-HSP (B) on a preparative reversed-phase HPLC Vydac 214TP1022 column. The bars indicate the fractions pooled for lyophilization. The dashed lines show the concentration of acetonitrile in the eluting solvent. For details, see Material and Methods.

25 Fig. 8 shows reversed-phase HPLC on a Vydac 214TP54 column of purified, glycosylated r-HSP (A) and r-HSP (B). The dashed lines show the concentration of acetonitrile in the eluting solvent.

Fig. 9 shows mass spectra of purified r-HSP (A and B) and
30 glycosylated r-HSP (C and D). Fig. A and Fig. C show the original mass spectrum of r-HSP and glycosylated r-HSP, respectively. Fig. B and Fig. D show the reconstructed mass

spectrum for r-HSP and glycosylated r-HSP on the basis of Fig. A and Fig. C.

EXAMPLE

MATERIAL AND METHODS

5 General methods

Standard DNA techniques were used as previously described [29]. Synthetic oligonucleotides were prepared on an automatic DNA synthesizer (380B, Applied Biosystems) using commercially available reagents. DNA sequence determinations were performed
10 by the dideoxy chain-termination technique [30]. Polymerase chain reactions (PCR) were performed on a DNA Thermal Cycler (Perkin Elmer Cetus) using a commercial kit (GeneAmp, Perkin Elmer Cetus).

PCR cloning of HSP

15 The first trefoil domain of HSP was isolated by a PCR reaction in which 1 μ g human genomic DNA (Clontech, Palo Alto, CA, USA) was used as a template. The reaction mixture contained 100 pmole each of the forward primer 1 (GGCTGAGCCCCCATAACAG) (SEQ ID NO:4) and reverse primer 2 (TGGAACACCAGGGGAC) (SEQ ID NO:5)
20 (Fig. 3) and was carried out in a 100 μ l volume. The cycle was : 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. After 30 cycles a final cycle was performed in which the 72°C step was maintained for 10 min. The PCR product, a 115 bp fragment, was isolated by electrophoresis on a 2% agarose gel.

25 The 115 bp PCR fragment was digested with DdeI and then ligated to a 31 bp duplex formed from the oligonucleotides (GAGAAACCCTCCCCCTGCCAGTGCTCCAGGC) (SEQ ID NO:6) and (TCAGCCTGGAGCACTGGCAGGGGGAGGGTTTCTC). The ligation product was amplified by PCR using forward primer 3
30 (GCTGAGAGATTGGAGAAGAGAGAGAAACCCTCCCCCT) (SEQ ID NO:7) and reverse primer 2. The 3' part of primer 3 is identical to the

N-terminal encoding part of the HSP gene and the 5' part of primer 3 is identical to the C-terminal encoding part of the hybrid leader gene (Fig. 3). In-frame fusion of the hybrid leader gene and the first trefoil domain from HSP was obtained by overlay extension PCR [31]. The product was digested with EcoRI and AvaII and isolated as a 360 bp DNA fragment.

The second trefoil domain of HSP was PCR-cloned from human genomic DNA as described for the first domain by replacing primers 1 and 2 with forward primer 4 (TGCCTCATGGAGGTCTC) (SEQ ID NO:8) and reverse primer 5 (AGCACCATGGCACTTCAAAG) (SEQ ID NO:9) (Fig. 3). Reverse primer 5 introduces a NcoI site as a silent mutation. The PCR product, a 115 bp fragment, was isolated and digested with DdeI and NcoI resulting in a 91 bp fragment. To this fragment were ligated two synthetic duplexes. The first, encoding the amino acid sequence between the two trefoil domains, consisted of the oligonucleotides (GTCCCCCTGGTGTTCACCCCCCTCCCAAAGCAAGAGTCGGATCAGTGCCTCATGGAGGTC) (SEQ ID NO:10) and (TGAGACCTCCATGACGCACTGATCCGACTCTTGCTTTGGGAGGGGGTGGAACACCAGGG) (SEQ ID NO:11). The second, a 46 bp NcoI - XbaI fragment encoding the C-terminal part of HSP, consisted of the oligonucleotides (CATGGTGCTTCTTCCCGAACTCTGTGGAAGACTGCCATTACTAAGT) (SEQ ID NO:12) and (CTAGACTTAGTAATGGCAGTCTTCCACAGAGTTCGGGAAGAAGCAC) (SEQ ID NO:13). After AvaII digestion a 195 bp AvaII - XbaI fragment was isolated.

A DNA construct encoding the hybrid leader fused in-frame to the entire HSP gene was obtained by ligation of the 360 bp EcoRI - AvaII fragment and the 195 bp AvaII - XbaI fragment described above to the 2.7 kb EcoRI - XbaI fragment from vector pTZ19R [32]. This construct was then transformed into *E. coli* strain MT-172 (r⁻, m⁻) by selection for resistance to ampicillin. DNA sequencing of the resulting plasmid, KFN-1843, showed that the correct construction had been obtained.

Construction of the HSP secreting yeast strain

Plasmid KFN-1843 described above was digested with EcoRI and XbaI. The resulting 558 bp fragment was isolated and ligated to the 9.3 kb NcoI - XbaI fragment and the 1.6 kb NcoI - EcoRI 5 fragment both from the yeast expression vector pMT-636. Plasmid pMT-636 is derived from the S. cerevisiae - E. coli shuttle vector CPOT [25,33] by deletion of the 0.4 kb HpaI - NruI fragment from the Leu-2 gene. The ligation mixture was transformed into E. coli strain MT-172, and the HSP expression 10 plasmid, KFN-1847, was isolated (Fig. 4). Plasmid pKFN-1847 was transformed into S. cerevisiae strain MT-663 by selection for growth on glucose as the sole carbon source. One transformant, KFN-1852, was selected for fermentation.

Fermentation

15 The transformant described above was cultivated at 30°C for 3 days in yeast peptone dextrose (YPD) medium [40] supplied with additional yeast extract (60 g/l). An OD 650 nm value of 52 was reached at the end of the fermentation.

Purification of r-HSP

20 The concentration of r-HSP in the yeast fermentation broth and fractions obtained during the purification was measured by analytical HPLC. Aliquots (usually 50-200 µl) were injected onto a Vydac 214TP54 reverse-phase C4 HPLC column (0.46 x 25 cm) equilibrated at 30°C at a flow rate of 1.5 ml/min with 0.1% 25 (v/v) TFA in 5% (v/v) acetonitrile. The concentration of acetonitrile in the eluting solvent was raised to 65% (v/v) over 30 min. Absorbance was measured at 280 nm. The peaks eluting at 15.6 min. and 16.1 min. (Fig. 5) was found by mass spectrometry analysis to represent glycosylated r-HSP and 30 unglycosylated r-HSP, respectively. The peptides were quantified using a calibrated PSP sample as standard as both

peptides contain two Trp and two Tyr out of 106 amino acid residues.

From a 10 litre fermentor, 8 litres of fermentation broth was isolated by centrifugation at 3,000 rpm for 10 min. The supernatant was concentrated to 0.9 litre using an Amicon ultrafiltration unit (RA 2000) equipped with an Amicon spiral ultrafiltration cartridge type S1Y3, MW cutoff 3,000 (Product No. 540620). The pH was adjusted to 1.7 and the conductivity in the resulting concentrated sample was measured to 4.7 mS.

- 10 The sample was pumped onto a Fast Flow S-Sepharose (Pharmacia) column (5 x 11 cm) with a flow rate of 40 ml/h. Previous to the application, the column was equilibrated in 50 mM formic acid buffer, pH = 3.7. After application of the sample, the column was washed with 500 ml of 50 mM formic acid buffer, pH = 3.7.
- 15 The peptides were eluted from the column by a linear gradient between 1.5 litres of 50 mM formic acid buffer, pH = 3.7 and 1.5 litres of 50 mM formic acid buffer, pH = 3.7 containing 0.6 M NaCl. Fractions of 10 ml was collected at a flow rate of 40 ml/h and the absorbance was measured at 280 nm. Fractions were
- 20 assayed for the content of r-HSP and glycosylated r-HSP in the HPLC-system previously described. The elution profile is shown in Fig. 6. Fractions corresponding to r-HSP (fract. Nos. 107-128) and glycosylated r-HSP (fract. Nos. 78-95), respectively, were pooled.
- 25 Glycosylated r-HSP and r-HSP were further purified by preparative HPLC chromatography. Pooled fractions (approx. 200 ml) were pumped onto a Vydac 214TP1022 C4 column (2.2 x 25 cm) equilibrated in 0.1% (v/v) TFA. The column was washed with 100 ml of 0.1% (v/v) TFA in 10% (v/v) MeCN. The peptides were
- 30 eluted at 25°C and at a flow rate of 5 ml/min with a linear gradient (650 ml) formed from MeCN/H₂O/TFA (10.0:89.9:0.1 v/v/v) and MeCN/H₂O/TFA (60.0:39.9:0.1 v/v/v). UV-absorption was monitored at 280 nm, and fractions corresponding to 10 ml were collected and analysed for the content of r-HSP or glycosylated

r-HSP. Fig. 7 shows the preparative HPLC purification of r-HSP (Fig. 7A) and glycosylated r-HSP (Fig. 7B). Fractions corresponding to the bars were pooled, and the volume reduced to 30% by vacuum centrifugation. From the two resulting pools, r-5 HSP and glycosylated r-HSP were isolated by lyophilization.

Characterization of r-HSP and glycosylated r-HSP

Amino acid composition analysis were carried out by hydrolysis of 50 μ g peptide with 6M HCl for 24 h at 110°C as previously described [6]; no correction for loss during hydrolysis was carried out. Amino acid sequence analysis was determined by automated Edman degradation using an Applied Biosystems Model 470A gas-phase sequencer [22]. Carbohydrate composition analysis was carried out by hydrolysis of 50 μ g peptide with 2M HCl for 1h, 2h and 4h at 100°C and monosaccharides were separated on a CarboPac PAI (Dionex, Sunnyvale, CA) column (4 x 250 mm) eluted with 14 mM NaOH. The monosaccharides were detected by pulsed amperometric detection (Dionex PAD-detector). The amount of monosaccharides was corrected to zero time of hydrolysis and calculated as nmol of monosaccharide per 20 nmol of peptide.

Mass spectrometry analysis was performed using an API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada). The triple quadrupole instrument has a mass-to-charge (m/z) range of 2400 and is fitted with a pneumatically assisted electrospray (also referred to as ion-spray) interface [23,24]. Sample introduction was done by a syringe infusion pump (Sage Instruments, Cambridge, MA) through a fused capillary (75 μ m i.d.) with a liquid flow-rate set at 0.5-1 μ l/min. The instrument m/z scale was calibrated with the singly-charged ammonium adduct ions of poly(propylene glycols) (PPG's) under unit resolution.

The accuracy of mass measurements was generally better than 0.02%.

RESULTS

Expression and purification

DNA fragments encoding the two trefoil domains of HSP were isolated by PCR from human genomic DNA using primers based on the published cDNA sequence [8]. The full length HSP gene was obtained from the PCR cloned fragments by addition of synthetic DNA fragments. The HSP gene was fused in-frame to a hybrid yeast leader sequence by overlay extension PCR [31] (Fig. 3). The hybrid leader is based on the mouse salivary amylase signal peptide [34] and the S. kluyveri α mating factor leader sequence [35] and is further modified near the Kex 2 cleavage site for efficient processing [36, 41].

The yeast expression plasmid pKFN-1847 contains the leader-HSP gene inserted between the S. cerevisiae triose phosphate isomerase promoter and terminator [37]. The expression vector (Fig. 4) also contains the Schizosaccharomyces pombe TPI gene (POT) [38].

The plasmid was transformed into the yeast strain MT-663, carrying a deletion in the TPI gene, by selecting for growth on glucose.

The expression level of r-HSP in the present yeast system is approx. 120 mg/l. As can be seen from Fig. 5, the yeast supernatant contains two forms of r-HSP; one eluting at $R_t = 15.6$ min. and one eluting at $R_t = 16.1$ min. These two forms were purified separately, and by using the analytical HPLC-system (Fig. 5), these two forms can be quantified individually during the different steps of the purification.

After the initial concentration of the yeast supernatant by ultrafiltration, the first purification step was cationic exchange chromatography on a Fast Flow S column. Fig. 6 shows the elution profile from the column including the amount of r-

HSP and glycosylated r-HSP determined in the fractions. A complete separation of the two forms of r-HSP was obtained in this step.

The fractions from the Fast Flows S column were pooled as indicated in Fig. 6, and the two peptides were further purified by preparative HPLC (Fig. 7). The r-HSP and glycosylated r-HSP were recovered from the fractions indicated in Fig. 7A and Fig. 7B by vacuum centrifugation and lyophilization. The purification is summarized in Table 1. The overall yield of r-HSP and glycosylated r-HSP from 8 litres of fermentation broth was 160 mg and 219 mg corresponding to 50% and 34%, respectively.

Characterization of r-HSP and glycosylated r-HSP

Fig. 8 shows the purity of r-HSP and glycosylated r-HSP as analysed by analytical HPLC. From these results none of the peptides looks completely pure. However, upon rechromatography of material eluting in the minor as well as the major peak, similar chromatograms were obtained for both peptides (results not shown). This seems to indicate that the double peak observed for both r-HSP and glycosylated r-HSP reflects an atypical behaviour of these peptides on reverse phase columns rather than impurities in the preparations.

Table 2 shows the amino acid sequencing results obtained on r-HSP and glycosylated r-HSP. The average repetitive yield was 94.4% (r-HSP) and 94.6% (glycosylated r-HSP), respectively. In both cases the first 40 residues of the two peptides were confirmed by the sequence analysis. In the glycosylated HSP, no PTH-a.a. was found in Edman degradation cycle No. 15. The HSP sequence from residue 15-17 (Asn-Arg-Thr) corresponds to a consensus sequence for N-glycosylation of Asn-15.

The carbohydrate composition analysis of glycosylated r-HSP showed the presence of 12.8 nmol mannose (Man) and 1.6 nmol of

N-acetyl glucosamine (GlcNAc) per nmol of r-HSP. By peptide mapping of r-HSP and glycosylated r-HSP in combination with mass spectrometry and sequencing analysis (results not shown), no other residue besides Asn-15 of the glycosylated r-HSP was found to be modified, i.e. no O-glycosylation was found.

In Fig. 9, the electro-spray mass spectrometry (ESMS) analysis is shown for r-HSP and glycosylated r-HSP. Fig. 9A and 9C are original mass spectra displaying characteristic series of multiply charged protonated ions always observed in ESMS spectra of proteins. Fig. 9B and 9D are the corresponding computer reconstructed mass spectra from which the molecular weight of individual components may be read directly. As can be seen from Fig. 9B, the MW found for r-HSP is 11961.5 ± 2 which is in very good agreement with a calculated mass of 11961.3. Fig. 9D shows the reconstructed ion spray mass spectrum of the glycosylated r-HSP. From the sequence analysis and the carbohydrate composition analysis, it is known that only Asn-15 is glycosylated and that only two monosaccharide residues, mannose and N-acetyl glucosamine, occur in the glycosylated form of r-HSP. From these results in combination with the mass spectrometry data, it is possible to deduce the different glycosylated forms of r-HSP (Table 3).

Molecular weights corresponding to two series of carbohydrate side chains can be deduced from the combination of carbohydrate composition data and ISMS-data, namely $(\text{GlcNAc})_2(\text{Hex})_{10-15}$ and $(\text{Hex})_{13-17}$ (Table 3). As mannose is the only hexose in the glycosylated r-HSP, and as Asn-15 is the only glycosylated residue, it seems reasonable to conclude that the structure of the glycosylation site is $\text{Asn}-(\text{GlcNAc})_2-(\text{Man})_{10-15}$. The observed $\text{Asn}-(\text{Hex})_{13-17}$ forms are thus most likely to arise from fragmentation in the mass spectrometer, by which the two GlcNAc residues lose an acetyl group and are converted into two hexoses.

The structure of Asn-(GlcNAc)₂-(Man)₁₀₋₁₅ has previously been reported as high mannose type of N-glycosylation for other peptides and proteins expressed in yeast [26].

Table 1

5 Purification of r-hSP and glycosylated r-hSP from yeast supernatant

STEP	Volume [ml]	Amount [mg]		Yield [%]	
		r-hSP	glycosylated r-hSP	r-hSP	glycosylated r-hSP
Yeast supernatant	8000	320	640	100	100
Ultrafiltration	900	207	405	65	63
Ion exchange chromatography	Pool 1	160	275		43
	Pool 2	220	182	57	
Prep HPLC	Pool 1	54	219		34
	Pool 2	80	160	50	

Table 2

Amino acid sequence analysis of r-hSP and glycosylated r-hSP

Cycle No.	PTH-a.a.	Yield (pmol)	
		r-hSP	glycosylated r-hSP
1	Glu	4304	8853
2	Lys	6925	8292
3	Pro	6027	12837
4	Ser	2890	5602
5	Pro	4336	8802
6	(Cys)	ND	ND
7	Gln	3388	5689
8	(Cys)	ND	ND
9	Ser	1279	2417
10	Arg	1876	2523
11	Leu	2277	4290
12	Ser	877	1790
13	Pro	1545	2963
14	His	517	574
15	Asn	1202	0*
16	Arg	959	1471
17	Thr	978	2172
18	Asn	1066	1509
19	(Cys)	ND	ND
20	Gly	836	1857
21	Phe	993	1958
22	Pro	843	1839
23	Gly	785	2049
24	Ile	640	1400
25	Thr	589	1454
26	Ser	274	621
27	Asp	581	1391
28	Gln	445	952
29	(Cys)	ND	ND
30	Phe	623	1562
31	Asp	483	1210
32	Asn	369	823
33	Gly	359	885
34	(Cys)	ND	ND
35	(Cys)	ND	ND
36	Phe	422	1094
37	Asp	268	783
38	Ser	127	324
39	Ser	145	394
40	Val	298	827

ND: Not determined

*: No trace of PTH-Asn or PTH-Asp was seen in cycle No. 15 of glycosylated r-hSP.

Table 3

Mass analysis of glycosylated r-hSP

Structure	Calculated MW	MW found by ESMS (Fig.9D)
hSP + 2 GlcNAc + 10 Man	13989.1	13989.5
hSP + 2 GlcNAc + 11 Man	14151.2	14151.0
hSP + 2 GlcNAc + 12 Man	14313.4	14313.5
hSP + 2 GlcNAc + 13 Man	14475.5	14475.0
hSP + 2 GlcNAc + 14 Man	14639.7	14639.5
hSP + 2 GlcNAc + 15 Man	14799.8	14801.5
hSP + 13 Man	14069.1	14072.0
hSP + 14 Man	14231.3	14232.5
hSP + 15 Man	14393.4	14393.0
hSP + 16 Man	14555.5	14557.5
hSP + 17 Man	14717.7	14720.0

REFERENCES

- [1] Thim, L. (1989) FEBS Lett. 250, 85-90.
- [2] Suemori, S., Lynch-Devaney, K. and Podolsky, D.K. (1991) Proc. Natl. Acad. Sci. USA 88, 11017-11021.
- 5 [3] Jakowlew, S.B., Breathnach, R., Jeltsch, J.M., Masiakowski, P. and Chambon, P. (1984) Nucleic Acids Res. 12, 1861-2878.
- [4] Prud'homme, J.-F., Fridlansky, F., Le Cunff, M., Atger, M., Mercier-Bodart, C., Pichon, M.-F. and
10 Milgrom, E. (1985) DNA 4, 11-21.
- [5] Rio, M.C., Bellocq, J.P., Daniel, J.Y., Tomasetto, C., Lathe, R., Chenard, M.P., Batzenschlager, A. and Chambon, P. (1988) Science 241, 705-708.
- [6] Thim, L., Thomsen, J., Christensen, M. and Jørgensen, K.H. (1985) Biochim. Biophys. Acta 827, 410-418.
15
- [7] Rose, K., Savoy, L.-A., Thim, L., Christensen, M. and Jørgensen, K.H. (1989) Biochim. Biophys. Acta 998, 297-300.
- [8] Tomasetto, C., Rio, M.-C., Gautier, C., Wolf, C., Hareuveni, M., Chambon, P. and Lathe, R. (1990) EMBO J. 9, 407-414.
20
- [9] Hoffmann, W. (1988) J. Biol. Chem. 263, 7686-7690.
- [10] Hauser, F. and Hoffmann, W. (1991) J. Biol. Chem. 266, 21306-21309.
- 25 [11] Hauser, F., Roeben, C. and Hoffmann, W. (1992) J. Biol. Chem. 267, 14451-14455.

- [12] Jørgensen, K.H., Thim, L. and Jacobsen, H.E. (1982) Regul. Peptides 3, 207-219.
- [13] Thim, L., Jørgensen, K.H. and Jørgensen, K.D. (1982) Regul. Peptides 3, 221-230.
- 5 [14] Rasmussen, T.N., Raabjerg, L., Poulsen, S.S., Thim, L. and Holst, J.J. (1992) Histochemistry 98, 113-119.
- [15] Frandsen, E.K., Jørgensen, K.H. and Thim, L. (1986) Regul. Peptides 16, 291-297.
- [16] Frandsen, E.K. (1988) Regul. Peptides 20, 45-52.
- 10 [17] Rasmussen, T.N., Raabjerg, L., Poulsen, S.S., Thim, L. and Holst, J.J. (1992) Am. J. Physiol. (in press)
- [18] Jørgensen, K.D., Diamant, B., Jørgensen, K.H. and Thim, L. (1982) Regul. Peptides 3, 231-243.
- [19] Hoosein, N.M., Thim, L., Jørgensen, K.H. and Brattain, M.G. (1989) FEBS Lett. 247, 303-306.
- 15 [20] Wright, N.A., Poulson, R., Stamp, G.W., Hall, P.A., Jeffery, R.E., Longcroft, J.M., Rio, M.-C., Tomasetto, C. and Chambon, P. (1990) J. Pathol. 162, 279-284.
- 20 [21] Rio, M.-C., Chenard, M.-P., Wolf, C., Marcellin, L., Tomasetto, C., Lathe, R., Bellocq, J.P. and Chambon, P. (1991) Gastroenterology 100, 375-379.
- [22] Thim, L., Hansen, M.T. and Soerensen, A.R. (1987) FEBS Lett. 212, 307-312.
- 25 [23] Bruins, A.P., Covey, T.R. and Henion, J.D. (1987) Anal. Chem. 59, 2642-2646.

- [24] Covey, T.R., Bonner, R.F., Shushan, B.I. and Henion, J.D. (1988) Rapid Commun. Mass Spectrom. 2, 249-256.
- [25] Thim, L., Hansen, M.T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G. and Fiil, N.P. (1986) Proc. Natl. Acad. Sci. USA 83, 6766-6770.
- [26] Poulter, L. and Burlingame, A.L. (1990) in: Methods in Enzymology (McCloskey, J.A., ed.) 193, 661-689. Academic Press, Inc., San Diego, CA.
- [27] Gajhede, M., Thim, L., Jørgensen, K.H. and Melberg, S.G. (1992) Proteins: Structure, Function, and Genetics 13, 364-368.
- [28] Carr, M.D. (1992) Biochemistry 31, 1998-2004.
- [29] Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [30] Sanger, F., Micklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
- [31] Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 61-68.
- [32] Mead, D. A., Szczesna-Skorupa, E. and Kemper, B. (1986) Prot. Engin. 1, 67-74.
- [33] Kawasaki, G. (1984) 12th International Conference on Yeast Genetics and Molecular Biology, Sept. 17-24, 1984, Edinburgh, Scotland, Abstr. P15.

- [34] Hagenbüchle, O., Tosi, M., Schibler, U., Bovey, R., Wellauer, P. K., and Young, R. A. (1981) Nature 289, 643-646.
- [35] Egel-Mitani, M. and Hansen, M. T. (1987) Nucl. Acids
5 Res. 15, 6303-6304.
- [36] Christiansen, L. and Norris, K., personal communication.
- [37] Alber, T. and Kawasaki, G. (1982) J. Mol. Appl. Genet. 1, 419-434.
- 10 [38] Russell, P. R. (1985) Gene 40, 125-130.
- [39] Jeltsch, J. M., Roberts, M., Schatz, C., Garnier, J. M., Brown, A. M. C., and Chambon, P. (1987) Nucl. Acids. Res. 15, 1401-1414.
- [40] Sherman, F., Fink, G.R. and Hicks, J.B. (1981)
15 Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, New York.
- [41] Norris, K., Norris, F. and Bjoern, S.E. (1990) International Patent Application WO 90/10075.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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- (I) TELEX: 37304

(ii) TITLE OF INVENTION: Human Spasmolytic Polypeptide in Glycosylated Form

(iii) NUMBER OF SEQUENCES: 14

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 106 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Glu	Lys	Pro	Ser	Pro	Cys	Gln	Cys	Ser	Arg	Leu	Ser	Pro	His	Asn	Arg
1				5				10					15		
Thr	Asn	Cys	Gly	Phe	Pro	Gly	Ile	Thr	Ser	Asp	Gln	Cys	Phe	Asp	Asn
			20					25					30		

29

Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val Pro Trp Cys Phe His
 35 40 45

Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val Met Glu Val Ser Asp
 50 55 60

Arg Arg Ash Cys Gly Tyr Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser
 65 70 75 80

Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu Val Pro Trp Cys Phe
 85 90 95

Phe Pro Asn Ser Val Glu Asp Cys His Tyr
 100 105

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 563 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 77..553

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
- (B) LOCATION: 77..235

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 236..553

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT 60

ATAAAGGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC 109
 Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
 -53 -50 -45

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG 157
 Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
 -40 -35 -30

30

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn -25 -20 -15	205
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA GAG AAA CCC TCC CCC TGC Val Ala Met Ala Glu Arg Leu Glu Lys Arg Glu Lys Pro Ser Pro Cys -10 -5 1 5	253
CAG TGC TCC AGG CTG AGC CCC CAT AAC AGG ACG AAC TGC GGC TTC CCT Gln Cys Ser Arg Leu Ser Pro His Asn Arg Thr Asn Cys Gly Phe Pro 10 15 20	301
GGA ATC ACC AGT GAC CAG TGT TTT GAC AAT GGA TGC TGT TTC GAC TCC Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn Gly Cys Cys Phe Asp Ser 25 30 35	349
AGT GTC ACT GGG GTC CCC TGG TGT TTC CAC CCC CTC CCA AAG CAA GAG Ser Val Thr Gly Val Pro Trp Cys Phe His Pro Leu Pro Lys Gln Glu 40 45 50	397
TCG GAT CAG TGC GTC ATG GAG GTC TCA GAC AGA AGA AAC TGT GGC TAC Ser Asp Gln Cys Val Met Glu Val Ser Asp Arg Arg Asn Cys Gly Tyr 55 60 65 70	445
CCG GGC ATC AGC CCC GAG GAA TGC GCC TCT CCG AAG TGC TGC TTC TCC Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser Arg Lys Cys Cys Phe Ser 75 80 85	493
AAC TTC ATC TTT GAA GTG CCA TGG TGC TTC TTC CCG AAC TCT GTG GAA Asn Phe Ile Phe Glu Val Pro Trp Cys Phe Phe Pro Asn Ser Val Glu 90 95 100	541
GAC TGC CAT TAC TAAGTCTAGA Asp Cys His Tyr 105	563

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
-53 -50 -45 -40

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
-35 -30 -25

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
-20 -15 -10

31

Arg Leu Glu Lys Arg Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu
-5 1 5 10

Ser Pro His Asn Arg Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp
15 20 25

Gln Cys Phe Asp Asn Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val
30 35 40

Pro Trp Cys Phe His Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val
45 50 55

Met Glu Val Ser Asp Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro
60 65 70 75

Glu Glu Cys Ala Ser Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu
80 85 90

Val Pro Trp Cys Phe Phe Pro Asn Ser Val Glu Asp Cys His Tyr
95 100 105

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCTGAGCCC CCATAACAG

19

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGGAACACC AGGGGAC

17

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAGAAACCCCT CCCCCTGCCA GTGCTCCAGG C

31

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCAGCCTGGA GCACTGGCAG GGGGAGGGTT TCTC

34

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

33

- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: synthetic

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCTGAGAGAT TGGAGAAGAG AGAGAAACCC TCCCCCT

37

- (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: synthetic

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGCGTCATGG AGGTCTC

17

- (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: synthetic

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGCACCATGG CACTTCAAAG

20

- (2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 60 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

34

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTCCCCCTGGT GTTCCACCC CCTCCCAAAG CAAGAGTCGG ATCAGTGGGT CATGGAGGTC

60

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGAGACCTCC ATGAGCACT GATCCGACTC TTGCTTTGGG AGGGGGTGGA AACACCAGGG

60

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CATGGTGCTT CTCCCGAAC TCTGTGGAAG ACTGCCATTA CTAAGT

46

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs

35

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTAGACTTAG TAATGGCAGT CTTCCACAGA GTTCGGGAAG AAGCAC

46

CLAIMS

1. Human spasmodic polypeptide (HSP) which has the amino acid sequence

Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu Ser Pro His Asn Arg
5 Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn
Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val Pro Trp Cys Phe His
Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val Met Glu Val Ser Asp
Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser
Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu Val Pro Trp Cys Phe
10 Phe Pro Asn Ser Val Glu Asp Cys His Tyr (SEQ ID NO:1)

or a functionally equivalent homologue thereof, characterized by being in glycosylated form.

2. HSP according to claim 1, characterized by being in N-glycosylated form.

15 3. HSP according to claim 1 or 2, which is glycosylated at Asn15.

4. HSP according to claim 3, in which the glycosylated side chain contains at least one hexose unit.

5. HSP according to claim 4, in which the glycosylated side
20 chain contains at least one mannose unit, preferably at least five mannose units, most preferably at least ten mannose units.

6. HSP according to claim 5, in which the glycosylated side chain contains 13-17 mannose units.

7. HSP according to claim 4, which in addition is glycosylated
25 with at least one unit of N-acetyl glucosamine (GlcNAc).

8. HSP according to claim 7, which is glycosylated with $(\text{GlcNAc})_2(\text{Man})_{10-15}$.
9. HSP according to claim 1, wherein Lys2 is replaced by Asn, Gln7 is replaced by Asn, Arg10 is replaced by Asn, Gly 20 is replaced by Thr or Ser, Gly23 is replaced by Asn, Ile 24 is replaced by Asn, Phe 36 is replaced by Asn, Asp 37 is replaced by Asn, Ser39 is replaced by Asn, Gln53 is replaced by Asn, Glu61 is replaced by Asn, Asp64 is replaced by Asn, Arg66 is replaced by Thr or Ser, Gly69 is replaced by Thr or Ser, Gly72 is replaced by Asn, Ile 89 is replaced by Thr or Ser, Pro98 is replaced by Asn or Val101 is replaced by Thr or Ser, or a combination of two or more of these substitutions.
10. HSP according to claim 9, wherein Asp64 is replaced by Asn, and wherein Arg66 is replaced by Thr or Ser.
11. HSP according to claim 9, in which the glycosylated Asn contains at least one hexose unit.
12. HSP according to claim 11, which is glycosylated with at least one mannose unit, preferably at least five mannose units, most preferably at least ten mannose units.
13. HSP according to claim 12, which is glycosylated with 13-17 mannose units.
14. HSP according to claim 11, which in addition is glycosylated with at least one unit of N-acetyl glucosamine (GlcNAc).
15. HSP according to claim 14, which is glycosylated with $(\text{GlcNAc})_2(\text{Man})_{10-15}$.
16. A variant of a spasmolytic polypeptide which is a fragment of human spasmolytic polypeptide (HSP) or porcine spasmolytic polypeptide (PSP) comprising at least one trefoil domain.

17. A variant according to claim 16, which comprises at least a sequence of amino acids from position 8 to 46 or from position 58 to 95.

18. A variant according to claim 16 or 17, wherein Asn15 is substituted by another amino acid, e.g. Asp or Glu.

19. A variant according to claim 16 or 17, wherein Thr17 is substituted by another amino acid except Ser, e.g. Ala.

20. A variant according to claim 16 or 17, wherein Arg10 is replaced by Asn, Gly 20 is replaced by Thr or Ser, Gly23 is replaced by Asn, Ile 24 is replaced by Asn, Phe 36 is replaced by Asn, Asp 37 is replaced by Asn, Ser39 is replaced by Asn, Glu61 is replaced by Asn, Asp64 is replaced by Asn, Arg66 is replaced by Thr or Ser, Gly69 is replaced by Thr or Ser, or Gly72 is replaced by Asn, or a combination of two or more of these substitutions.

21. A method of preparing a spasmolytic polypeptide in at least 60% glycosylated form, wherein a host cell transformed with a DNA fragment encoding a spasmolytic polypeptide and capable of providing glycosylation of said spasmolytic polypeptide is cultured under conditions permitting production of said spasmolytic polypeptide and recovering the resulting spasmolytic polypeptide from the culture.

22. A method according to claim 21, wherein the host cell is a fungal cell such as a yeast cell, e.g. a strain of Saccharomyces cerevisiae, or a filamentous fungus cell, e.g. a strain of Aspergillus, such as Aspergillus oryzae.

23. A pharmaceutical composition comprising HSP according to any of claims 1-15 together with a pharmaceutically acceptable carrier or excipient.

24. A pharmaceutical composition comprising a variant spasmolytic polypeptide according to any of claims 16-20 together with a pharmaceutically acceptable carrier or excipient.

5 25. Use of HSP according to any of claims 1-15 for the manufacture of a medicament for the prophylaxis or treatment of gastrointestinal disorders.

26. Use of a variant spasmolytic polypeptide according to any of claims 16-20 for the manufacture of a medicament for the
10 prophylaxis or treatment of gastrointestinal disorders.

Trefoil family of peptides










Peptide	Number of trefoil domains	Species
Intestinal Trefoil Factor (ITF)		Rat Human
Breast cancer associated peptide (pS2)		Human
Spasmolytic polypeptide (hSP) (PSP) (mSP)	 	Human Porcine Mouse
Spasmolysins (xP1)		Frog
(xP4)	   	Frog

Fig. 1

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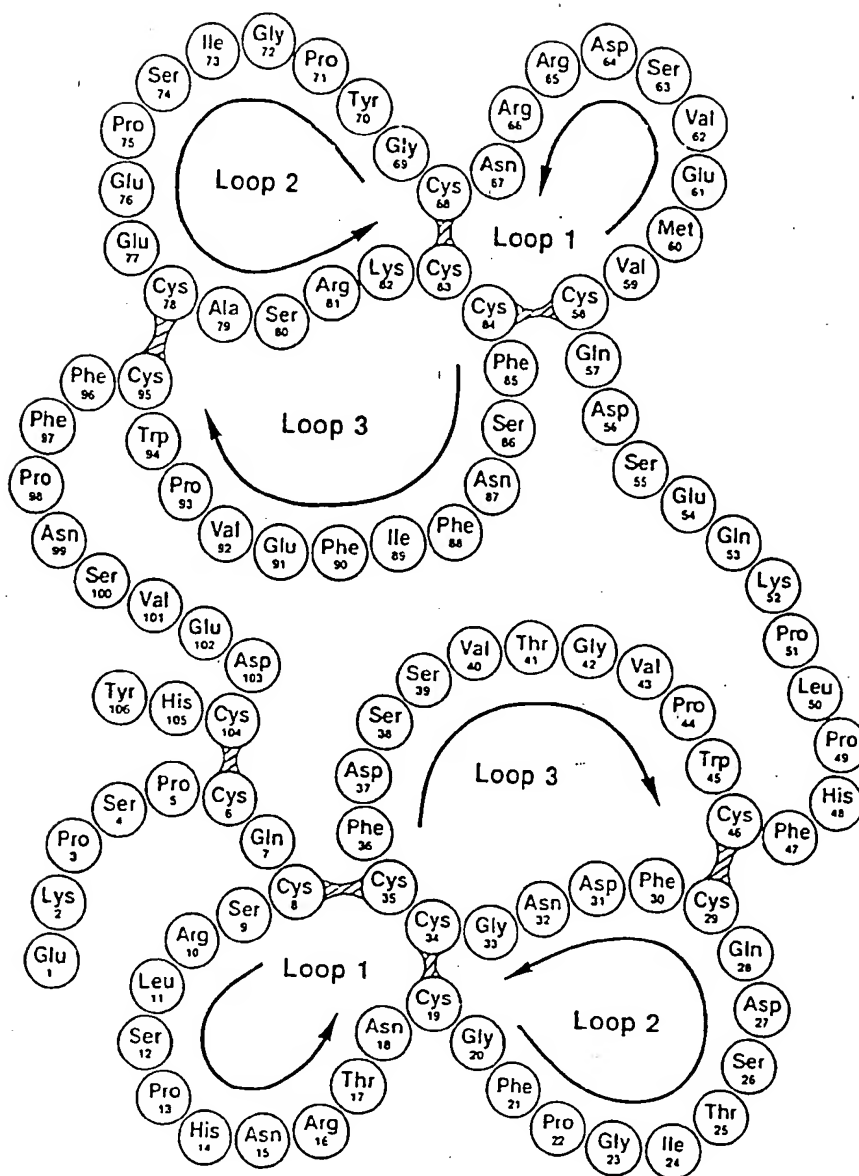


Fig. 2

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EcoRI

GAATTCATTCAAGAATAGTTCAAACAAGAAGATTACAAACTATCAATTTTCATACACAAT

ATAAACGACCAAAAAGAATGAAGGCTGTTTTCTTGGTTTTTGTCTTGATCGGATTCTGCTG
 M K A V F L V L S L I G F C W

GGCCCAACCAGTCACTGGCGATGAATCATCTGTTGAGATTCCGGAAGAGTCTCTGATCAT
 A Q P V T G D E S S V E I P E E S L I I

CGCTGAAAACACCACTTTGGCTAACGTCGCCATGGCTGAGAGATTGGAGAAGAGagagaa
 A E N T T L A N V A M A E R L E K R E K

Dde I 1
 accctccccctgccagtgctccaggcTGAGCCCCCATAACAGGACGAACTGCGGCTTCCC
 P S P C Q C S R L S P H N R T N C G F P

TGGAATCACCAGTGACCAGTGTTTTGACAATGGATGCTGTTTCGACTCCAGTGTCAGTGG
 G I T S D Q C F D N G C C F D S S V T G

AvaII 2
 Ggtccccctggtgtttccacccccctcccaaagcaagagtcggatcagtgcgatcatgagcgt
 V P W C F H P L P K Q E S D Q C V M E V

Dde I
 cTCAGACAGAAGAACTGTGGCTACCCGGGCATCAGCCCCGAGGAATGCGCCTCTCGGAA
 S D R R N C G Y P G I S P E E C A S R K

5 Nco I
 GTGCTGCTTCTCCAACCTTCATCTTTGAAGTGccatggtgcttcttcccgaactctgtgga
 C C F S N F I F E V P W C F F P N S V E

Xba I
 agactgccattactaagtCTAGA
 D C H Y

Fig. 3

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hSP expression vector

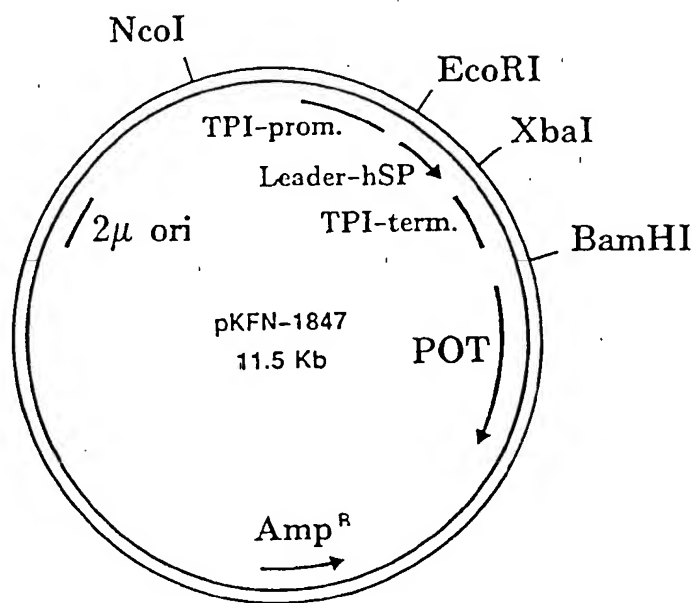


Fig. 4

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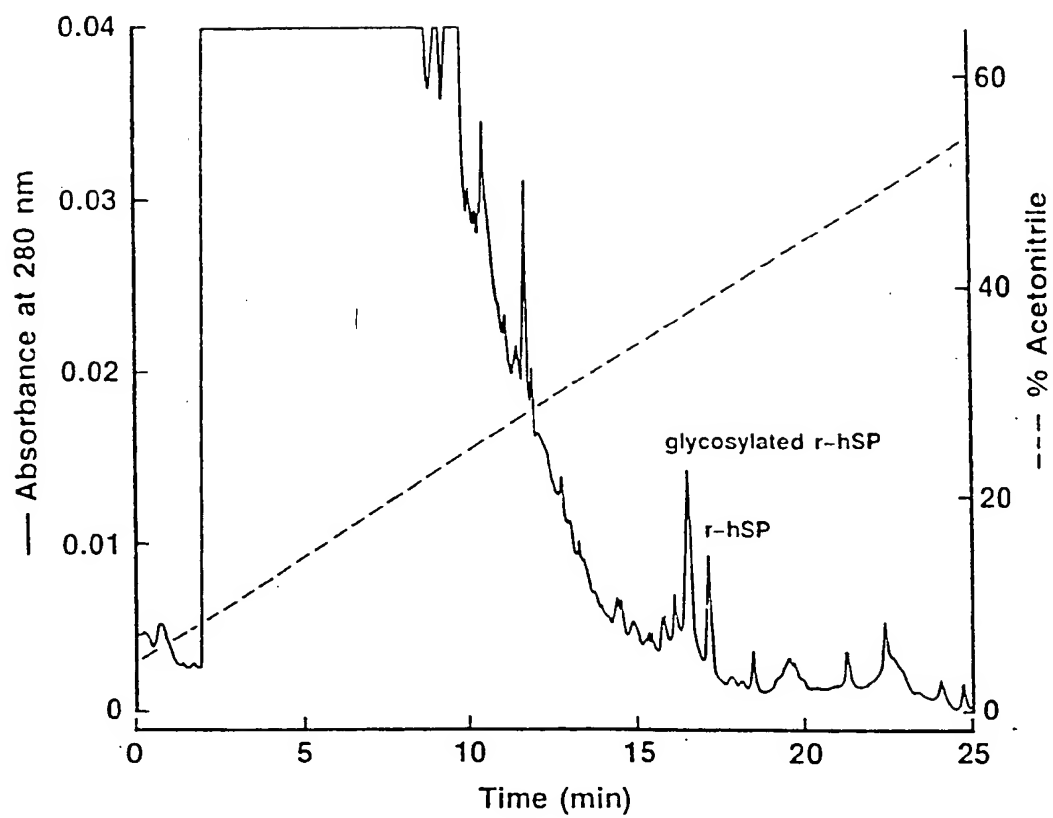


Fig. 5

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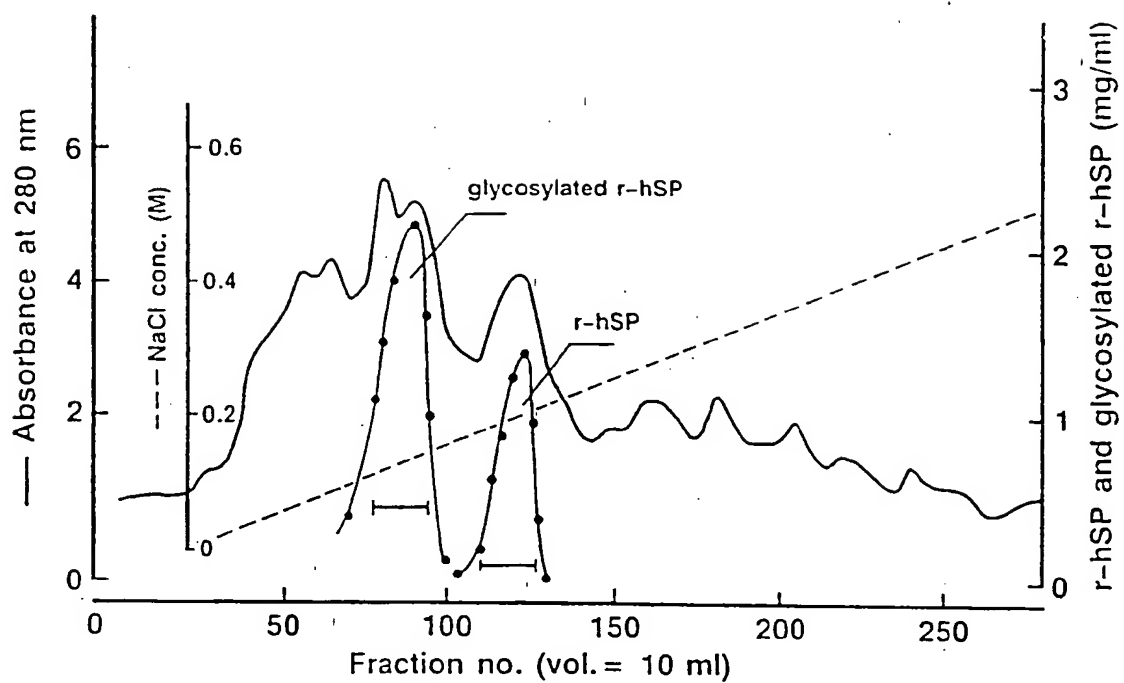


Fig. 6

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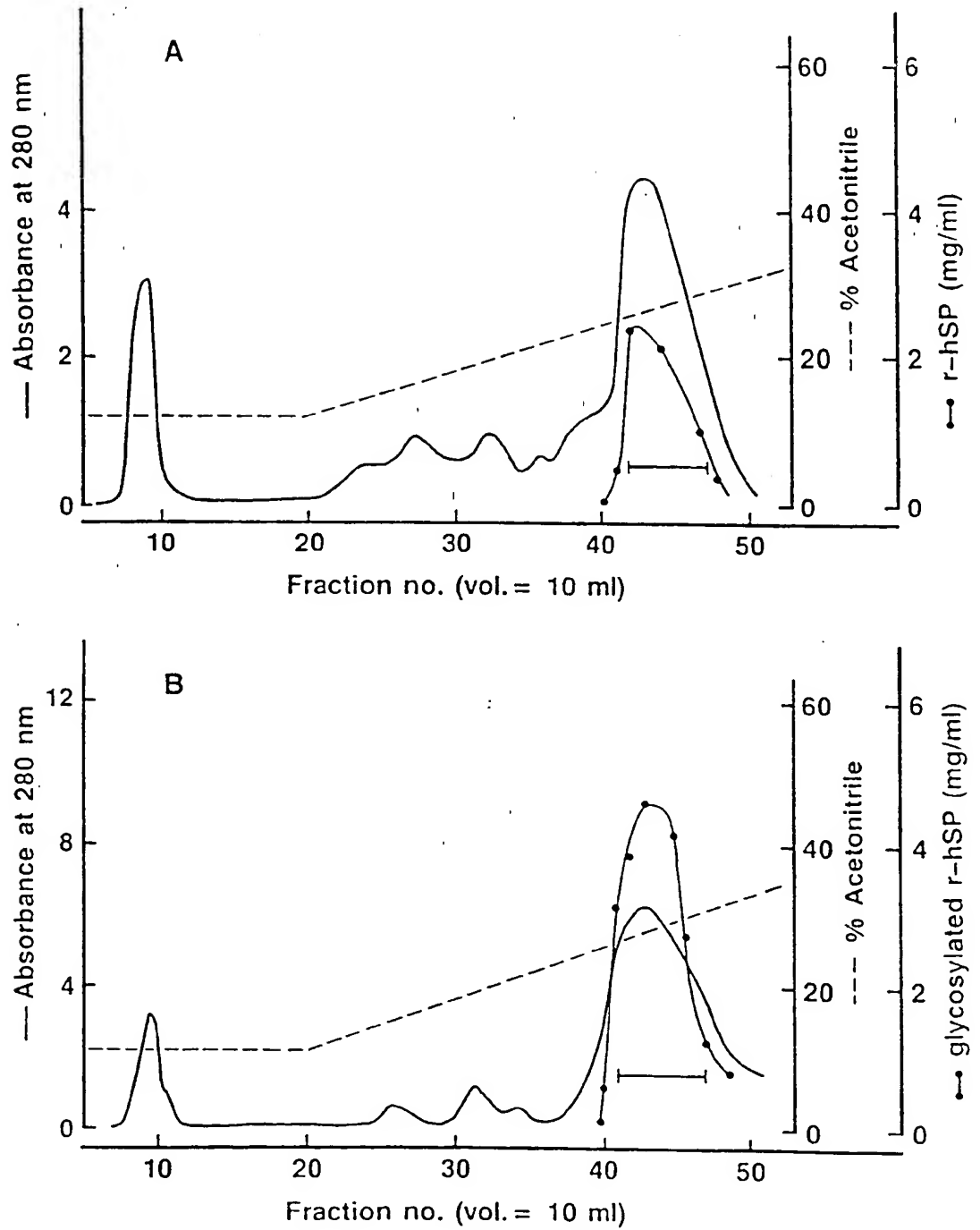


Fig. 7

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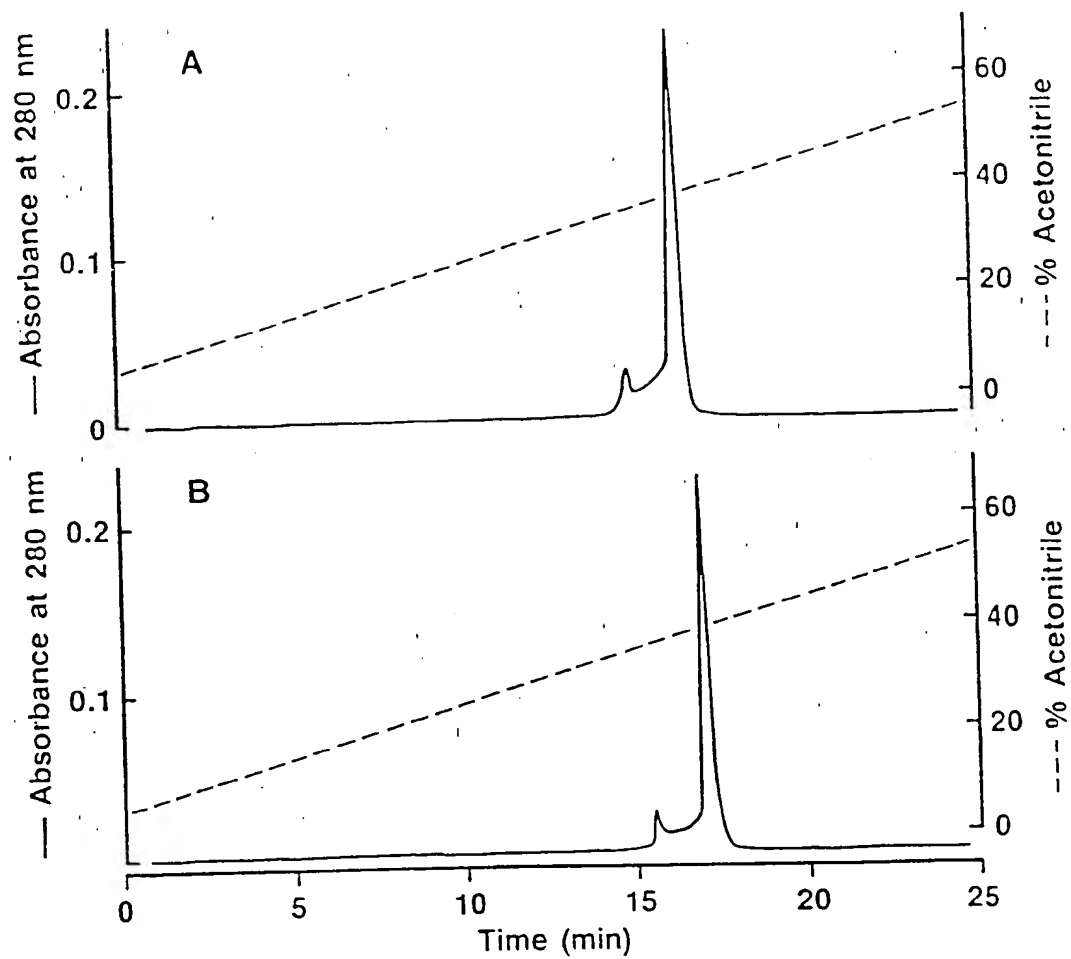


Fig. 8

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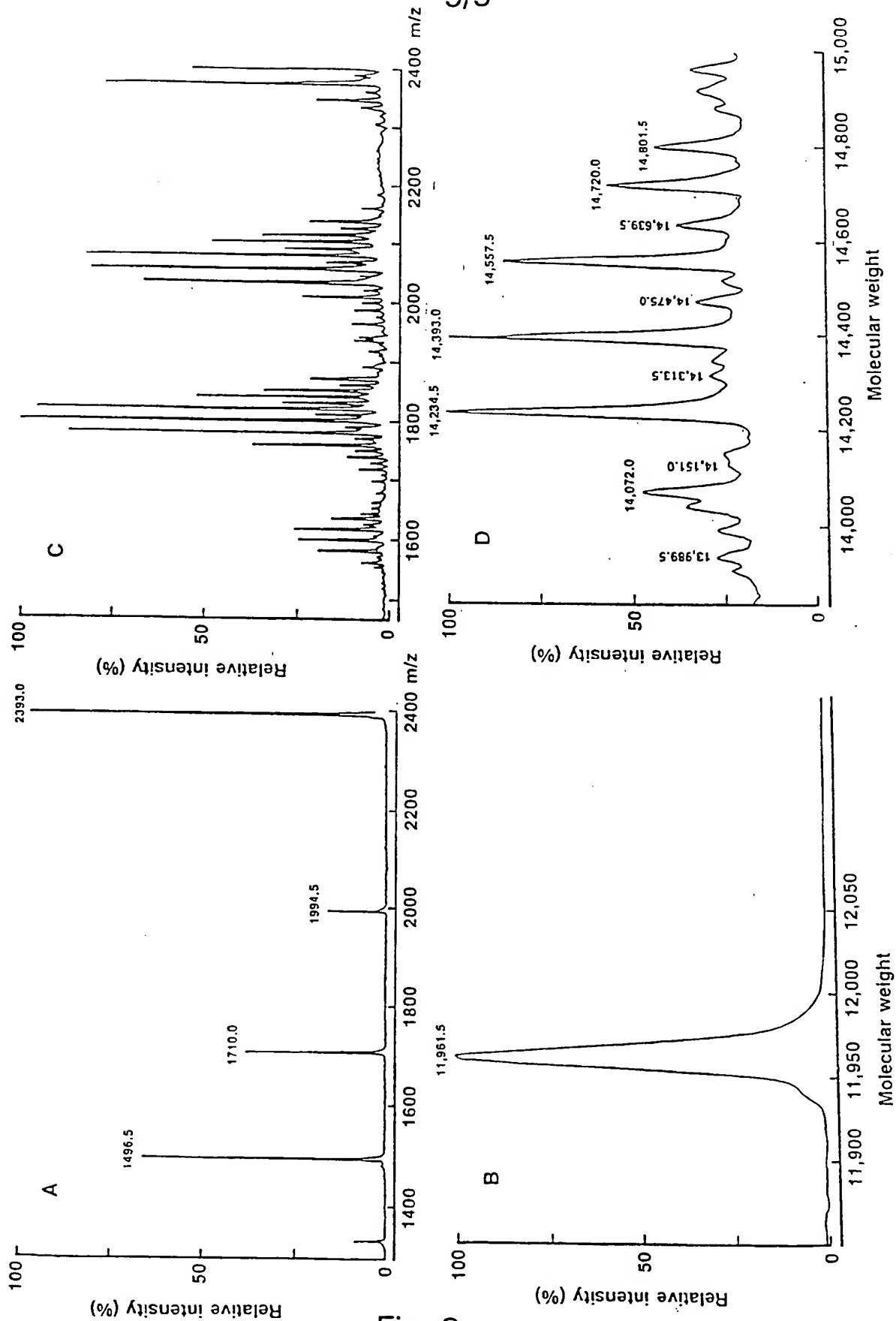


Fig. 9

REPLACEMENT SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00037

A. CLASSIFICATION OF SUBJECT MATTER

IPC : C07K 15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EMBL, PIRONLY, GENESEQ, SWISSPROT, MEDLINE, BIOSIS, WPI, CLAIMS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The EMBO Journal, Volume 9, No 2, 1990, C Tomasetto et al., "hSP, the domain-duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma" page 407 - page 414	1-26
	--	
X	Dialog Information Services, File 155, MEDLINE, Dialog acc.no. 05506304, Thim L. et al: "The amino acid sequence of pancreatic spasmolytic polypeptide", Biochim Biophys Acta Mar 1 1985, 827 (3) p410-8	1-26
	-- -----	

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

27 June 1994

Date of mailing of the international search report

01 -07- 1994

Name and mailing address of the ISA/
Swedish Patent Office
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00037

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet 206

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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